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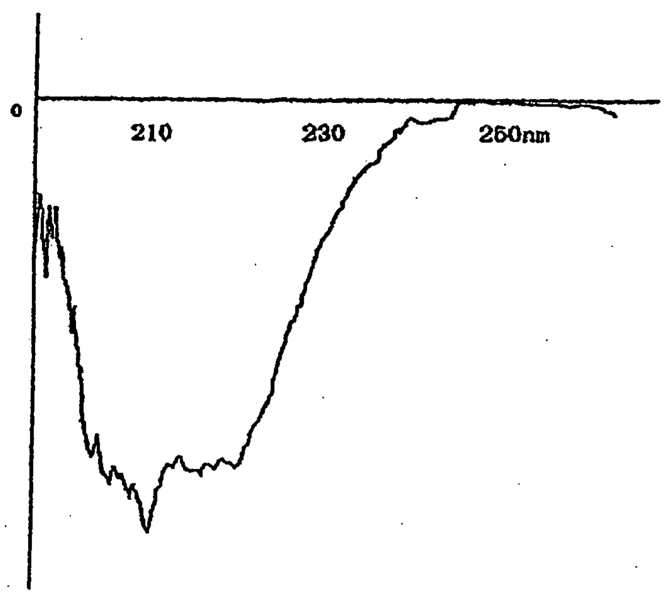
(54) Title: USES OF SPATIAL CONFIGURATION TO MODULATE PROTEIN FUNCTION



(57) Abstract: This invention provides a set of methods for modulating protein spatial configuration. First, select the amino-acid codon for encoding the target protein according to host codon usage. Second, choose combinations which can modulate the spatial configuration and construct into different vectors which can transfect a series of hosts. Third, choose the vector promoter by monitoring a combination of base pairs after combining the code sequence of the promoter and the target protein. Finally, choose the appropriate expression host to express the target protein, refold and purify, measure the activity and spatial configuration.

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USES OF SPATIAL CONFIGURATION TO MODULATE PROTEIN FUNCTION

The application disclosed herein claims priority of U.S. Serial No. 60/498,449, filed August 28, 2003; U.S. Serial No. 60/498,785, filed August 28, 2003; and U.S. Serial No. 60/498,923, filed August 28, 2003. This application claims priority of Indian Application No. 279/MUM/2004, filed March 5, 2004, and Indian Application No. 280/MUM/2004, filed March 5, 2004. The contents of the preceding applications are hereby incorporated in their entireties by reference into this application.

Throughout this application, various publications are referenced. Disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

BACKGROUND OF THE INVENTION

The completion of the human genome project verified the therapeutic effects of many genes, and some of them have been developed into therapeutic proteins, but most of them cannot be controlled by gene or protein techniques in the art. They cannot be correctly translated into proteins which maintain the whole therapeutic effects possessed by their genes. The biggest obstacle on the road to successful protein translation is the correct protein-folding. The field of research on how to obtain a protein with efficient spatial configuration is filled with competition.

Changing the spatial configuration of proteins without disturbing amino acid sequence may change functions of certain proteins. For example, some proteins with abnormal 3-dimensional structure can cause diseases in humans and animals, such as: bovine spongiform encephalopathy (BSE), Alzheimer's Disease, cystic fibrosis, familial hypercholestrolacemia, familial amyloid disease, certain carcinoma or cataract. These diseases also have been

called folding-diseases". The "Prion" protein causes BSE and can infect normal proteins and transmit among them.

During the research of protein structure, most researchers consider that the most important part in retrieving the correct spatial structure of proteins are the techniques of denaturation and refolding. Masses of literature reported improvement in refolding associated with various chaperons or reverse micelles, etc. Many secretion expression vectors have been developed to allow those proteins expressed in more natural environments, but all these efforts only result in an increase in the yields of proteins, not in qualitative changes.

DETAILED DESCRIPTION OF THE FIGURES

Figure 1. Circular Dichroism spectrum of Infergen®
Spectrum range: 250nm - 190nm
Sensitivity: 2 m°/cm
Light path: 0.20 cm
Equipment: Circular Dichroism J-500C
Samples :contain 30μg/ml IFN-con1, 5.9 mg/ml of NaCl and 3.8 mg/ml of Na_2PO_4, pH7.0.

Figure 2. Circular Dichroism spectrum of rSIFN-co
Spectrum range: 250nm - 190nm
Sensitivity: 2 m°/cm
Light path: 0.20 cm
Equipment: Circular Dichroism J-500C
Samples :contain 30μg/ml rSIFN-co, 5.9 mg/ml of NaCl and 3.8 mg/ml of Na_2PO_4, pH7.0.

Figure 3. Comparison of Inhibition Effects of Different Interferons on HBV Gene Expression

Figure 4A-1. Curves of Changes of Body Temperature in Group A (5 patients)
This figure is the record of body temperature changes of 5 patients in Group A.

Figure 4A-2. Curves of Changes of Body Temperature in Group A (6 patients)
This figure is the record of body temperature changes of the other 6 patients in Group A.

Figure 4B-1. Curves of Changes of Body Temperature in Group B (5 patients)
This figure is the record of body temperature changes of 5 patients in Group B.

Figure 4B-2. Curves of Changes of Body Temperature

in Group B (5 patients)
This figure is the record of body temperature changes of the other 5 patients in Group B.

Figure 5. rsIFN-co Crystal I

Figure 6. rsIFN-co Crystal II

Figure 7. The X-ray Diffraction of rsIFN-co Crystal

DETAILED DESCRIPTION OF THE INVENTION

This invention provides a set of methods for modulating protein spatial configuration. First, select the amino-acid codon for encoding the target protein according to host codon usage. Second, choose combinations which can modulate the spatial configuration and construct into different vectors which can transfect a series of hosts. Therefore, an appropriate vector with appropriate host may be chosen. Third, choose the vector promoter by monitoring a combination of base pairs after combining the code sequence of the promoter and the target protein. Finally, choose the appropriate expression host to express the target protein, refold and purify, measure the activity and spatial configuration.

This invention discovered that during the protein-constructing process, the variation of codon that encodes the amino acid of target protein, the difference of choosing vectors, the modulation of the promoter and the selection of host expression vector, even conditions of denaturation and renaturation, agents etc. are all adjustable factors for modulating the spatial configuration of target proteins. Accordingly, modulation of the spatial configuration of proteins to obtain new functions and to improve activity is the result of systematic analysis.

This invention provides a method for modulating the function of proteins without changing the primary amino acid sequence of said protein comprising steps of: a) altering the codon usage of said protein; b) expressing the protein using the altered codon to obtain purified protein; and c) comparing the expressed protein with altered codon usage to one without, wherein an increase in function or identification of new function indicates that the function of the protein has been modulated.

In an embodiment, the altered codon usage results in high expression of said protein.

This invention also provides a method for preparing protein with enhanced or new functions without changing the primary amino acid sequence of said protein comprising steps of: a) altering the codon usage of said protein; b) expressing the protein using the altered codon to obtain purified protein; and c) comparing the expressed protein with altered codon usage to one without, wherein an increase in function or identification of new function indicates that a protein with enhanced and new function has been prepared.

In an embodiment, the altered codon usage results in high expression of said protein. This invention also provides the protein prepared by the above method. In an embodiment, the protein has unique secondary or tertiary structure.

This invention further provides a synthetic gene with altered codon, which, when expressed, produces enhanced or new functions. In an embodiment, the invention provides a vector comprising the gene. In a further embodiment, this invention provides an expression system comprising the gene. In yet a further embodiment, this invention provides a host cell comprising the gene.

This invention also provides a process for production of a protein of enhanced function or new function comprising introducing an artificial gene with selected codon preference into an appropriate host, culturing said introduced host under appropriate conditions for the expression of said protein, and harvesting the expressed protein.

This invention provides the above process, wherein the artificial gene is operatively linked to a vector. In an embodiment, the process comprises extraction of the protein from fermentation broth, or collection of the inclusion body, and denaturation and renaturation of the harvested

protein.

This invention also provides the protein produced by any of the above processes.

This invention provides a composition comprising any of the above proteins and a suitable carrier. This invention further provides a pharmaceutical composition comprising any of the above produced proteins and a pharmaceutically acceptable carrier.

One significance of this invention is that it modulates the spatial configuration of protein during the process of translating genes with therapeutic effects into proteins which possess functions originating from the genes, or functions not seen in proteins produced using traditional techniques, or even with improved activity compared with those existing proteins.

Taking the interferon as an example, construct the gene of human IFN-α into reverse transcriptive expression vector to produce PDOR-INF-α expression vector, then transfect 2.2.15 cell. HBsAg and HBeAg in the culturing supernatant of cell is measured. The results indicate that the suppression rate of rSIFN-co to HBsAg was 62% and 67.7% to HBeAg, but the recombinant interferon protein produced by gene recombination techniques do not have the effect in vitro. In addition, the experiment of constructing the human INF-α2 expression vector using the reverse transcriptive viral vector and transfecting it into HIV cell strain-A3.01 proved that IFN-α2 can completely restrain the replication and transcript of HIV-DNA. However, the effect of interferon is limited in the treatment of HIV disease.

This invention will be better understood from the examples which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more

fully in the claims which follow thereafter.

Example 1:

CONFORMATION RECONSTRUCTION OF IFN-CONL

rSIFN-co is a new interferon molecule constructed according to conservative amino acids in human IFN-α subtype with genetic engineering methods. The interferon has been described in United States Patent Nos. 4,695,263 and 4,897,471, and has been proven in literature and patents to have broad-spectrum interferon activity with strong antiviral, anti-tumor and natural cell-killing effects.

The DNA coding sequence was redesigned according to *E. Coli*. codon usage by first constructing an insert into pHY-4 vector, mediating down-stream expression with P_{BAD} promoter, then choosing E. Coli. as host. The high-purity products are gained by denaturation with 6 mol/L guanidine hydrochloride → renatured with 4 mol/L arginine → purified with Cu^{2+}-chelating affinity chromatography after POROS HS/M cation exchange chromatography.

The comparison test of duplicates of hepatitis B virus DNA and secretion of HBsAg and HBeAg inhibition between rSIFN-co and IFN-con_1 proved that rSIFN-co has the effect of inhibiting the secretion of HBsAg and HBeAg which is not possessed by IFN-conl. In another test, the HBV core/ pregenomic(C/P) promoter and associate cis-acting element were placed upstream of luciferase-encoding plasmid. This reporter construct was transfected into HpeG2 cells. The cells were treated with different interferons and luciferase reporter gene expression was measured. Results show that rSIFN-co can suppress 68% of luciferase reporter gene expression; whereas IFN-conl and IFN-α2b only suppress 35% and 27% of it. Therefore, the suppression effect of rSIFN-co on HBcAg has been obviously improved.

Meanwhile, circular dichroism spectrum also proved there are differences in the secondary structure of rSIFN-co by comparison with IFN-con1.

The following are those comparison experiments in detail:

1) Comparison of circular dichroism spectrum

Address: The Center of Analysis and Test in Sichuan University

Apparatus: J-500C Circular Dichroism equipment (spectrum range: 250-190nm / sensibility : 2 m^0/cm / light path: 0.2cm. (See Figure 1 and Figure 2.)

2) rSIFN-co inhibits HBV-DNA duplication and secretion of HBsAg and HBeAg.

Materials

Solvent and Dispensing Method: Add 1ml saline into each vial, dissolve, and mix with MEM culture medium at different concentrations. Mix on the spot.

Control drugs: IFN-α2b (Intron A) as lyophilized powder, purchased from Schering Plough. 3×10^6U each, mix to 3×10^6IU/ml with culture medium; INFERGEN (liquid solution), purchased from Amgen, 9μg, 0.3ml each, equal to $9X10^6$IU, and mix with $9X10^6$IU/ml culture medium preserve at 4℃; 2.2.15 cell: 2.2.15 cell line of hepatoma (Hep G2) cloned and transfected by HBV DNA, constructed by Mount Sinai Medical Center.

Reagent: MEM powder, Gibco American Ltd. cattle fetal blood serum, HycloneLab American Ltd. G-418(Geneticin); MEM dispensing, Gibco American Ltd.; L-Glutamyl, imported and packaged by JING KE Chemical Ltd.; HBsAg and HBeAg solid-phase radioimmunoassay box, Northward Reagent Institute of Chinese Isotope Ltd.; Biograncetina, Northern China Medicine; and Lipofectin, Gibco American Ltd.

Experimental goods and equipment: culture bottle, Denmark Tunclon™; 24-well and 96-well culture board, Corning American Ltd.; Carbon Dioxide hatching box, Shel-Lab American Ltd.; MEM culture medium 100ml: 10% cattle fetal blood serum, 0.03% Glutamine, G418 380μg/ml, biograncetina 50U/ml.

Method:

2.2.15 cell culture: Add 0.25% pancreatic enzyme into culture box with full of 2.2.15 cell. Digest at 37°C for 3 minutes and add culture medium to stop digestion and disperse the cells. Reproduce with a ratio of 1:3. They will reach full growth in 10 days.

Toxicity test: Set groups of different concentrations and a control group in which cells are not acted on with medicine. Digest cells, and dispense to a 100,000 cell/ml solution. Inoculate to 96-well culture board, 200μl per well. Culture at 37°C for 24h with 5% CO_2. Test when simple cell layer grows.

Dispense rSIFN-co to 1.8×10^7IU/ml solution then prepare a series of solutions diluted at two-fold gradients. Add into 96-well culture board, 3 wells per concentration. Change the solution every 4 days. Test cytopathic effect by microscope after 8 days. Fully destroy as 4, 75% as 3, 50% as 2, 25% as 1, zero as 0. Calculate average cell lesions and inhibition rates at different concentrations. Calculate TC50 and TC0 according to the Reed Muench method.

$$\text{TC50} = \text{Antilog}\ (B + \frac{50-B}{A-B} \times C)$$

A=log >50% medicine concentration; B=log<50% medicine concentration; C=log dilution power

Inhibition test for HBeAg and HBsAg: Separate into positive and negative HBeAg and HBsAg contrast groups, cell contrast groups and medicine concentration groups.

inoculate 700,000 cells/ml of 2.2.15 cell into 6-well culture board, 3 ml per well, culture at 37°C for 24h with 5% CO_2, then prepare 5 gradiently diluted solutions with 3-fold as the grade (Prepare 5 solutions, each with a different protein concentration. The concentration of Solution 2 is 3 times lower than that of Solution 1, the concentration of Solution 3 is 3 times lower than that of Solution 2, etc.) 4.5×10^6IU/ml, 1.5×10^6IU/ml, 0.5×10^6IU/ml, $0.17\times10^6$1U/ml, and $0.056\times10^6$1U/ml, 1 well per concentration, culture at 37°C for 24h with 5% CO_2. Change solutions every 4 days using the same solution. Collect all culture medium on the 8th day. Preserve at -20□ Repeat test 3 times to estimate HBsAg and HBeAg with solid-phase radioimmunoassay box (Northward Reagent Institute of Chinese Isotope Ltd.). Estimate cpm value of each well with a γ- accounting machine.

Effects calculation: Calculate cpm mean value of contrast groups and different-concentration groups and their standard deviation, P/N value such as inhibition rate, IC50 and SI.

1) Antigen inhibition rate (%) = $\frac{A-B}{A}\times 100$

A = cpm of control group; B = cpm of test group;

2) Counting the half-efficiency concentration of the medicine

Antigen inhibition IC50 = Antilog (B + $\frac{50-B}{A-B}$ × C)

A=log>50% medicine concentration; B=log<50□medicine concentration; C=log dilution power

3) SI of interspace-conformation changed rSIFN-co effect on HBsAg and HBeAg in 2.2.15 cell culture:

$$SI = \frac{TC50}{IC50}$$

4) Estimate the differences in cpm of each dilution degree from the control group using student *t* test

Southern blot: (1) HBV-DNA extract in 2.2.15 cell: Culture cell 8 days. Exsuction culture medium (Separate cells from culture medium by means of draining the culture medium.). Add lysis buffer to break cells, then extract 2 times with a mixture of phenol, chloroform and isoamyl alcohol (1:1:1), 10,000g centrifuge. Collect the supernatant adding anhydrous alcohol to deposit nucleic acid. Vacuum draw, re-dissolve into 20μlTE buffer. (2) Electrophoresis: Add 6XDNA loading buffer, electrophoresis on 1.5% agarose gel, IV/cm, at fixed pressure for 14-18h. (3) Denaturation and hybridization: respectively dip gel into HCl, denaturaion buffer and neutralization buffer. (4) Transmembrane: Make an orderly transfer of DNA to Hybond-N membrane. Bake, hybridize and expose with dot blot hybridization. Scan and analyze relative density with gel-pro software. Calculate inhibition rate and IC50.

Results

Results from Tables 1, 2 and 3 show: After maximum innocuous concentration exponent culturing for 8 days with 2.2.15 cell, the maxima is $9.0 \pm 0\times10^6$IU/ml average inhibition rate of maximum innocuous concentration rSIFN-co to HBeAg is 46.0±5.25% (P<O□001), IC50 is $4.54\pm1.32\text{X}10^6$IU/ml, SI is 3.96; rate to HBsAg is 44.8± 6.6%, IC50 is $6.49\pm0.42\times10^6$IU/ml, SI is 2.77. This shows that rSIFN-co can significantly inhibit the activity of HBeAg and HBsAg, but that the IFN of the contrast group and INFERGEN cannot. It has also been proven in clinic that rSIFN-co can decrease HBeAg and HBsAg or return them to normal levels.

Table 1: Results of inhibition rate of rSIFN-co to HBsAg and HBeAg

First batch: (rSIFN-co)

Inhibition effect to HBeAg										
Concentration ($\times10^4$IU/ml)	First well	Second well	Third well	Inhibition rate			Average inhibition rate	Accumulation	1-Accumulation	Accumulated inhibition rate
				First well	Second well	Third well				
900	9026	8976	10476	0.436227	0.43935	0.345659	0.407079	0.945909	0.592921	0.614693546
300	9616	12082	10098	0.3993754	0.245347	0.369269	0.337997	0.5388299	1.254924	0.300392321
100	9822	16002	12800	0.386508	0.0005	0.2005	0.195836	0.200833	2.059088	0.08867188
33.33333	15770	19306	16824	0.014991	0	0	0.004997	0.0049969	3.054091	0.001633453
11.11111	19172	22270	18934	0	0	0	0	0	4.054091	0
Control	Cell	16010		Blank	0		Dilution	3	IC50	602.74446016

Inhibition effect to HBsAg										
Concentration ($\times10^4$IU/ml)	First well	Second well	Third well	Inhibition rate			Average inhibition rate	Accumulation	1-Accumulation	Accumulated inhibition rate
				First well	Second well	Third well				
900	7706	7240	7114	0.342155	0.381936	0.392693	0.372261	0.922258	0.627739	0.595006426
300	8856	7778	9476	0.2439816	0.336008	0.191053	0.257014	0.5499972	1.370724	0.286349225
100	10818	10720	10330	0.07649	0.084856	0.118149	0.093165	0.292983	2.27756	0.113977019
33.33333	10744	11114	10570	0.082807	0.051221	0.097661	0.07723	0.1998179	3.20033	0.058767408
11.11111	10672	9352	10810	0.088953	0.201639	0.077173	0.122588	0.122588	4.077742	0.02918541
Control	Cell	11714		Blank	0		Dilution	3	IC50	641.7736749

Second batch: (rSIFN-co)

Inhibition effect to HBeAg										
Concentration ($\times 10^4$IU/ml)	First well	Second well	Third well	Inhibition rate			Average inhibition rate	Accumulation	1-Accumulation	Accumulated inhibition rate
				First well	Second well	Third well				
900	7818	8516	9350	0.554378	0.514592	0.467054	0.512008	1.371181	0.487992	0.737521972
300	10344	10628	9160	0.4103967	0.394209	0.477884	0.427497	0.8591731	1.060496	0.447563245
100	12296	14228	13262	0.299134	0.18901	0.244072	0.244072	0.4316522	1.816423	0.19201839
33.33333	15364	17414	16188	0.124259	0.00741	0.77291	0.069653	0.1876045	2.74677	0.063933386
11.11111	17386	13632	15406	0.009006	0.222982	0.121865	0.117951	0.117951	3.628819	0.03148073
Control	Cell	16962		Blank	0		Dilution	3	IC50	365.9357846

Inhibition effect to HBsAg										
Concentration ($\times 10^4$IU/ml)	First well	Second well	Third well	Inhibition rate			Average inhibition rate	Accumulation	1-Accumulation	Accumulated inhibition rate
				First well	Second well	Third well				
900	5784	6198	5792	0.498265	0.462353	0.497571	0.486063	0.893477	0.513937	0.634835847
300	7150	8534	8318	0.379771	0.259715	0.278452	0.30598	0.4074138	1.207957	0.252210647
100	9830	11212	10210	0.147294	0.027412	0.11433	0.096345	0.101434	2.111612	0.04583464
33.33333	13942	12368	13478	0	0	0	0	0.0050891	3.111612	0.001632835
11.11111	12418	11634	11352	0	0	0.015267	0.005089	0.005089	4.106523	0.001237728
Control	Cell			Blank	0		Dilution	3	IC50	611.0919568

Third batch: (rSIFN-co)

Concentration (x104IU/ml)	First well	Second well	Third well	Inhibition rate			Average inhibition rate	Accumulation	1-Accumulation	Accumulated inhibition rate
				Inhibition effect to HBeAg						
				First well	Second well	Third well				
900	9702	9614	8110	0.428016	0.433204	0.521872	0.461031	1.316983	0.538969	0.709599543
300	8914	10032	8870	0.4744723	0.40856	0.477066	0.453366	0.8559525	1.085603	0.440859127
100	16312	12688	13934	0.038321	0.251975	0.178517	0.156271	0.402586	1.929332	0.172641621
33.33333	15080	12814	13288	0.110954	0.244547	0.216602	0.190701	0.2463153	2.738631	0.082519158
11.11111	21928	15366	15728	0	0.094093	0.072751	0.0055615	0.055615	3.683017	0.014875633
Control	Cell	17544		Blank	0		Dilution	3	IC50	382.0496935

Inhibition effect to HBsAg

Concentration (x104IU/ml)	First well	Second well	Third well	Inhibition rate			Average inhibition rate	Accumulation	1-Accumulation	Accumulated inhibition rate
				First well	Second well	Third well				
900	5616	6228	5346	0.496864	0.442035	0.521054	0.486651	0.763125	0.513349	0.597838293
300	8542	8590	7096	0.234725	0.230425	0.364272	0.276474	0.2764738	1.236875	0.182690031
100	11420	11360	11394	0	0	0	0	0	2.236875	0
33.33333	12656	11582	13110	0	0	0	0	0		0
11.11111	13142	12336	13342	0	0	0	0	0	4.236875	0
Control	Cell	11528		Blank	0		Dilution	3	IC50	694.7027149

HBeAg: Average IC50: 450.2434 SD: 132.315479

HBsAg: Average IC50: 649.1894 SD: 42.29580

Table 2: Results of inhibition rate of Intron A(IFN-α2b) to HBsAg and HBeAg

	Inhibition effect to HBeAg										
Concentration ($\times 10^4$IU/ml)	First well	Second well	Third well	Inhibition rate			Average inhibition rate	Accumulation	1-Accumulation	Accumulated inhibition rate
				First well	Second well	Third well				
300	14918	11724	9950	0	0.029711	0.176529	0.068747	0.068747	0.931253	0.068746724
100	14868	16890	15182	0	0	0	0	0	1.931253	0
33.33333	16760	21716	16400	0	0	0	0	0	2.931253	0
11.11111	20854	15042	16168	0	0	0	0	0	3.931253	0
3.703704	12083	12083	12083	0	0	0	0	0	4.931253	0
Control	Cell	17544		Blank	0		Dilution	3	IC50	FALSE
	Inhibition effect to HBsAg									
Concentration ($\times 10^4$IU/ml)	First well	Second well	Third well	Inhibition rate			Average inhibition rate	Accumulation	1-Accumulation	Accumulated inhibition rate
				First well	Second well	Third well				
300	9226	8196	9658	0.152489	0.247106	0.521054	0.1708	0.189295	0.8292	0.185857736
100	10946	10340	10828	0	0.050156	0.364272	0.018495	0.0184947	1.810705	0.010110817
33.33333	12250	12980	13934	0	0	0	0	0	2.810705	0
11.11111	12634	12342	12000	0	0	0	0	0	3.810705	0
3.703704	10886	10886	10886	0	0	0	0	0	4.810705	0
Control	Cell	10886		Blank	0		Dilution	3	IC50	FALSE

Table 3: Results of inhibition rate of Infergen to HBsAg and HBeAg

First batch: (Infergen)

				Inhibition effect to HBeAg							
Concentration ($\times 10^4$ IU/ml)	First well	Second well	Third well	Inhibition rate			Average inhibition rate	Accumulation	1-Accumulation	Accumulated inhibition rate
				First well	Second well	Third well				
900	14172	12156	17306	0.091655	0.220869	0	0.104175	0.306157	0.895825	0.254710274
300	13390	12288	16252	0.1417767	0.212409	0	0.118062	0.2019827	1.777764	0.102024519
100	14364	18834	14194	0.079349	0	0.090245	0.056531	0.083921	2.721232	0.029916678
33.33333	15722	16034	16340	0	0	0	0	0.0273897	3.721232	0.007306592
11.11111	17504	17652	14320	0	0	0.082169	0.02739	0.02739	4.693843	0.005801377
Control	Cell	15602		Blank	0		Dilution	3	IC50	FALSE
Inhibition effect to HBsAg										
Concentration ($\times 10^4$ IU/ml)	First well	Second well	Third well	Inhibition rate			Average inhibition rate	Accumulation	1-Accumulation	Accumulated inhibition rate
				First well	Second well	Third well				
900	12080	11692	12234	0	0.01275	0	0.00425	0.025163	0.39573	0.024647111
300	12840	11484	12350	0	0.030313	0	0.010104	0.0209125	1.985646	0.010422073
100	12894	14696	15086	0	0	0	0	0.010808	2.985646	0.003606955
33.33333	15032	12928	13020	0	0	0	0	0.0108081	3.985646	0.002704416
11.11111	11794	11984	11508	0.004137	0	0.028287	0.010808	0.010808	4.974837	0.002167838
Control	Cell	11843		Blank	0		Dilution	3	IC50	FALSE

Second batch: (Infergen)

Inhibition effect to HBeAg										
Concentration ($\times 10^4$ IU/ml)	First well	Second well	Third well	Inhibition rate			Average inhibition rate	Accumulation	1-Accumulation	Accumulated inhibition rate
				First well	Second well	Third well				
900	6278	6376	6408	0.200051	0.187564	0.183486	0.190367	0.274635	0.809633	0.253290505
300	7692	9092	6394	0.0198777	0	0.18527	0.068383	0.0842678	1.74125	0.046161005
100	8960	7474	8190	0	0.047655	0	0.015885	0.015885	2.725365	0.005794856
33.33333	8530	8144	9682	0	0	0	0	0	3.725365	0
11.11111	7848	7848	7848	0	0	0	0	0	4.725365	0
Control	Cell	7848		Blank	0		Dilution	3	IC50	FALSE

Inhibition effect to HBsAg										
Concentration ($\times 10^4$ IU/ml)	First well	Second well	Third well	Inhibition rate			Average inhibition rate	Accumulation	1-Accumulation	Accumulated inhibition rate
				First well	Second well	Third well				
900	12364	12268	12274	0.036171	0.043655	0.043187	0.041004	0.140162	0.958996	0.12751773
300	11590	12708	13716	0.0965076	0.009355	0	0.035287	0.0991581	1.923709	0.0490186
100	12448	13468	13982	0.029623	0	0	0.009874	0.063871	2.913834	0.02144964
33.33333	12616	11346	12444	0.016526	0.115529	0.029935	0.053996	0.0539965	3.859838	0.013796309
11.11111	12828	12828	12828	0	0	0	0	0	4.859838	0
Control	Cell	12828		Blank	0		Dilution	3	IC50	FALSE

Third batch: (Infergen)

Concentration ($\times 10^4$IU/ml)	First well	Second well	Third well	Inhibition effect to HBeAg: Inhibition rate			Average inhibition rate	Accumulation	1-Accumulation	Accu-mulated inhibition rate
				First well	Second well	Third well				
900	7240	6642	6158	0.064599	0.14186	0.204393	0.136951	0.217399	0.863049	0.201211735
300	11072	8786	6902	0	0	0.108269	0.03609	0.0804479	1.82696	0.042176564
100	7016	9726	7552	0.09354	0	0.024289	0.039276	0.044358	2.787683	0.015663017
33.33333	7622	8866	8676	0.015245	0	0	0.005082	0.0050818	3.782601	0.001341671
11.11111	7740	7740	7740	0	0	0	0	0	4.782601	0
Control	Cell	7740		Blank	0		Dilution	3	IC50	FALSE

Concentration ($\times 10^4$IU/ml)	First well	Second well	Third well	Inhibition effect to HBsAg: Inhibition rate			Average inhibition rate	Accumulation	1-Accumulation	Accu-Mulated inhibition rate
				First well	Second well	Third well				
900	11048	11856	11902	0.04775	0	0	0.015917	0.015917	0.984083	0.015916796
300	13454	12896	11798	0	0	0	0	0	1.984083	0
100	12846	13160	12546	0	0	0	0	0	2.984083	0
33.33333	12680	12458	12360	0	0	0	0	0	3.984083	0
11.11111	11602	11602	11602	0	0	0	0	0	4.984083	0
Control	Cell	11602		Blank	0		Dilution	3	IC50	FALSE

HBeAg: Average IC50: 0 SD: 0

HBsAg: Average IC50: 0 SD: 0

Example 2:

COMPARISON OF INHIBITORY EFFECTS OF DIFFERENT INTERFERONS ON HBV GENE EXPRESSION

Hepatitis B virus (HBV) DNA contains consensus elements for transactivating proteins whose binding activity is regulated by interferons. Treatment of HBV-infected hepatocytes with interferons leads to inhibition of HBV gene expression. The aim of the present study was to characterize the effects of different interferons on HBV regulated transcription. Using transient transfection of human hepatoma cells with reporter plasmids containing the firefly luciferase gene under the control of HBV-Enhancer (EnH) I, Enh II and core promoter, Applicant studied the biological activities of three different interferons on transcription.

Materials and Methods

1. Interferons: IFN-con1 (*Infergen*®), IFN-Hui-Yang (γSIFN-co) and IFN-beta 1b

2. Reporter plasmid: The DNA fragments containing HBV-Enhancer (EnH) I, Enh II and core promoter were prepared using PCR and blunt-end cloned into the Smal I site of the promoter- and enhancer-less firefly luciferase reporter plasmid pGL3-Basic (Promega, WI, USA). The resulting reporter plasmid was named as pGL3-HBV-Luc.

3. Cell Culture and DNA transfection: HepG2 cells were cultured in DMEM medium supplemented with 10% FBS and 100 U/ml penicillin and 100 ug/ml streptomycin. The cells were kept in 30°C, 5% CO2 incubator. The cells were transfected with pGL3-HBV-Luc reporter plasmid using Boehringer's Lipofectin transfection kit. After 18 hours, the medium containing transfection reagents was removed and fresh medium was added with or without interferons. The cells were kept in culture for another 48 hours.

4. Luciferase Assay: Forty-eight hours after the addition of interferon, the cells were harvested and cell lysis were

prepared. The protein concentration of cell lysates were measured using Bio-Rad Protein Assay kit. The luciferase activity was measured using Promega's Luciferase Reporter Assay Systems according to the instructions of manufacturer.

RESULTS

Expression of Luciferase Activity in Different Interferon - Treated Cell Lysates

No treatment	IFN-con1	IFN-Hui-Yang	IFN-beta 1b
100	48+8	29+6	64+10

This result shows that γSIFN-co inhibits most effectively on the expression of HBV gene expression.

Example 3:

SIDE EFFECTS AND CHANGES IN BODY TEMPERATURE WHEN USING γSIFN-co

There are usually more side effects to using interferon. The side effects include: nausea, muscle soreness, loss of appetite, hair loss, hypoleucocytosis (hypoleukmia; hypoleukocytosis; hypoleukia), and decrease in blood platelets, etc.

METHOD

Sample patients are divided into two groups. 11 patients in Group A were injected with 9μg Infergen®. 10 patients in Group B were injected with 9μg γSIFN-co. Both groups were monitored for 48 hours after injections. First monitoring was recorded 1 hour after injection, after that, records were taken every 2 hours.

Table 4 is the comparison of side effects between patients being injected with 9μg of Infergen® and 9μg of γSIFN-co.

Table 4. Side Effects

		γSIFN-co 9μg	Infergen® 9μg
		Person: n=10	Person: n=11
Body Systems	Reactions	Headcount	Headcount
In General	Feebleness	3	3
	Sole heat	1	
	Frigolability	3	4
	Decrease in leg strength		3
	Mild lumbago	2	1
	Body soreness	4	5
Central Nervous System/ Peripheral Nervous System	Headache	3	6
	Dizziness	2	11
	Drowsiness		3
Gastroenterostomy	Apoclesis	1	
	Celiodynia	1	
	Diarrhea	1	
Musculoskeletal system	Myalgia	1	2
	Arthralgia	2	
Respiratory system	Stuffy nose	1	
Paropsia	Swollen eyes		1

RESULTS

For those patients who were injected with γSIFN-co, the side effects were minor. They had some common symptoms similar to flu, such as: headache, feebleness, frigolability, muscle soreness, hidrosis, and arthralgia (arthrodynia; arthronalgia). The side effects of those patients whom were injected with Infergen® were worse than those were injected with γSIFN-co.

From Figures 4A-1, 4A-2, 4B-1, and 4B-2, it was obvious that the body temperatures of sample patients in Group A were higher than the patients in Group B. It also reflected that the endurance of γSIFN-co was much better than Infergen®.

Example 4:

CRYSTAL GROWTH of γSIFN-co AND TEST OF CRYSTALLOGRAPHY PARAMETER

Crystal of γSIFN-co. Two types of crystal were found after systematic trial and experiment. (See Figures 5-7)

1. Crystal Growth

Dissolve γSIFN-co protein with pure water (H2O) to 3mg/ml in density. Search crystallization by using Hampton Research Crystal Screen I and II which was made by Hampton Company. By using Drop Suspension Diffusion Method, liquid 500μl, drop 1 μl protein + 1 μl liquid, in 293K temperature. First 2 different types of small crystals were found as listed in Table 5.

Table 5. Screen of γSIFN-co Crystallin

Condition	I	II
Diluent	0.1M Tris-HCl PH=8.75	0.1M HEPES PH=7.13
Precipitant	17.5%(w/v) PEG550 MME	10%(w/v)PEG6K
Additives	0.1M NaCl	3%(v/v)MPD
Temperature	293K	293K
Crystal Size (mm)	0.2x0.2x0.1	0.6x0.02x0.02
Crystallogram	Figure 5	Figure 6

2. Data Collection and Processing

Crystal I was used to collect X-Ray diffraction data and preliminary analysis of crystallography. Testing of parameters was also completed. The diffraction data was collected under room temperature. Crystal I (Condition I) was inserted into a thin siliconized wall tube. By using BrukerAXS Smart CCD detector, light source CuKα (λ=1.5418Å) generated by Nonius FR591 X-ray generator. Light power 2000 KW (40 kv x 50mA), wave length 1.00Å, under explosion 60 second, Δφ=2°, the distance between crystal and detector was 50mm. Data was processed using Proteum Procedure Package by Bruker Company. For crystal diffraction pattern (partially), see Figure 7. See Table 6 for process results.

Table 6. Results of Crystallography Parameters

Parameters	
a (Å)	82.67
b (Å)	108.04
c (Å)	135.01
α (°)	90.00
β (°)	90.00
γ (°)	98.35

Space Group P2 or $P2_1$
Sharpness of separation 5 Å
Asymmetric molecule # 10
Dissolution 57.6%

In addition, there was no crystal growth of γSIFN-co based on previous publications. The closest result to the γSIFN-co was huIFN-a2b but the screen was very complicated. After seeding 3 times, crystal grew to 0.5x0.5x0.3mm, sharpness of separation was 2.9 Å, space group was $P2_1$. The crystals were also big, asymmetric molecule number was 6, and dissolution was about 60%.

1. A method for modulating the function of proteins without changing the primary amino acid sequence of said protein comprising steps of:
   a) altering the codon usage of said protein;
   b) expressing the protein using the altered codon to obtain purified protein; and
   c) comparing the expressed protein with altered codon usage to one without, wherein an increase in function or identification of new function indicates that the function of the protein has been modulated.

2. The method of claim 1, wherein the altered codon usage results in high expression of said protein.

3. A method for preparing protein with enhanced or new functions without changing the primary amino acid sequence of said protein comprising steps of:
   a) altering the codon usage of said protein;
   b) expressing the protein using the altered codon to obtain purified protein; and
   c) comparing the expressed protein with altered codon usage to one without, wherein an increase in function or identification of new function indicates that a protein with enhanced and new function has been prepared.

4. The method of claim 1, wherein the altered codon usage results in high expression of said protein.

5. The protein prepared by the method of claim 3 or 4.

6. The protein of claim 5 with unique secondary or tertiary structure.

7. A synthetic gene with altered codon which, when

8. A vector comprising the gene of claim 7.

9. An expression system comprising the gene of claim 7.

10. A host cell comprising the gene of claim 7.

11. A process for production of a protein of enhanced function or new function comprising introducing an artificial gene with selected codon preference into an appropriate host, culturing said introduced host under appropriate conditions for the expression of said protein, and harvesting the expressed protein.

12. The process of claim 11, wherein the artificial gene is operatively linked to a vector.

13. The process of claim 11, comprising extraction of the protein from fermentation broth, or collection of the inclusion body, and denaturation and renaturation of the harvested protein.

14. The protein produced by the process of any of claims 11-13.

15. A composition comprising the protein of claim 5, 6, or 14 and a suitable carrier.

16. A pharmaceutical composition comprising the produced protein of claim 5, 6, or 14 and a pharmaceutically acceptable carrier.

Figure 1

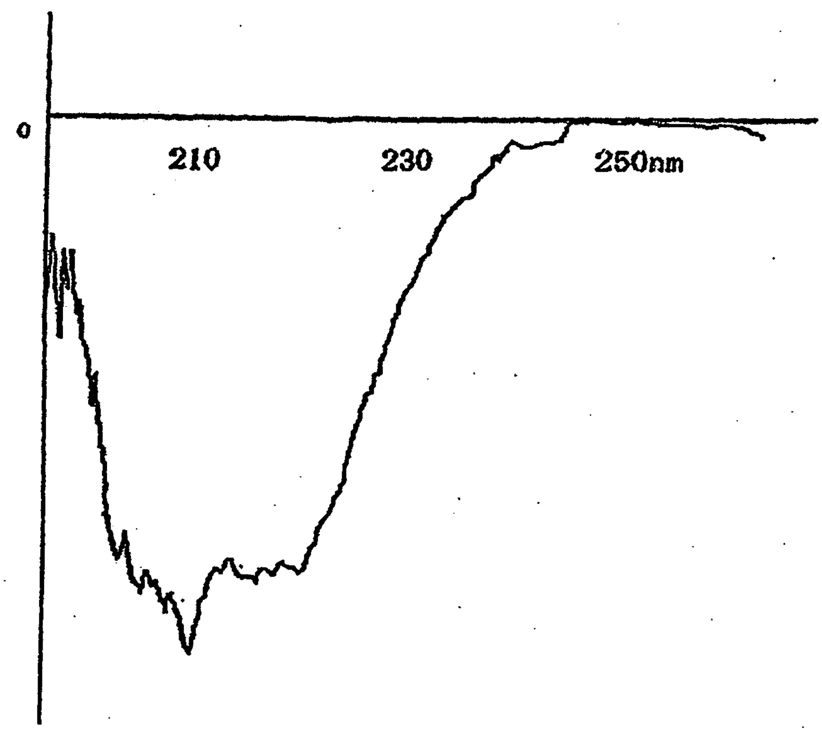
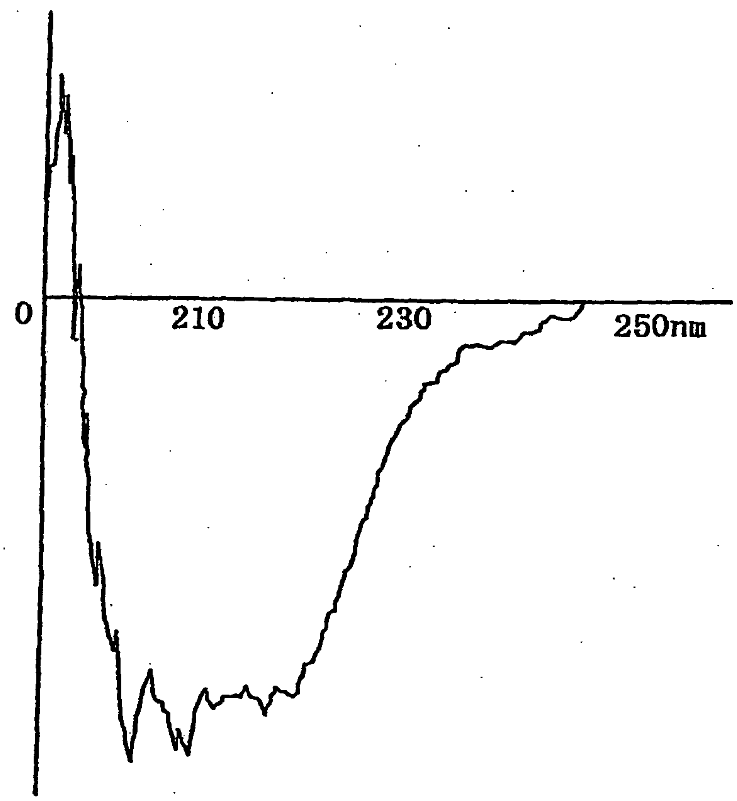


Figure 2



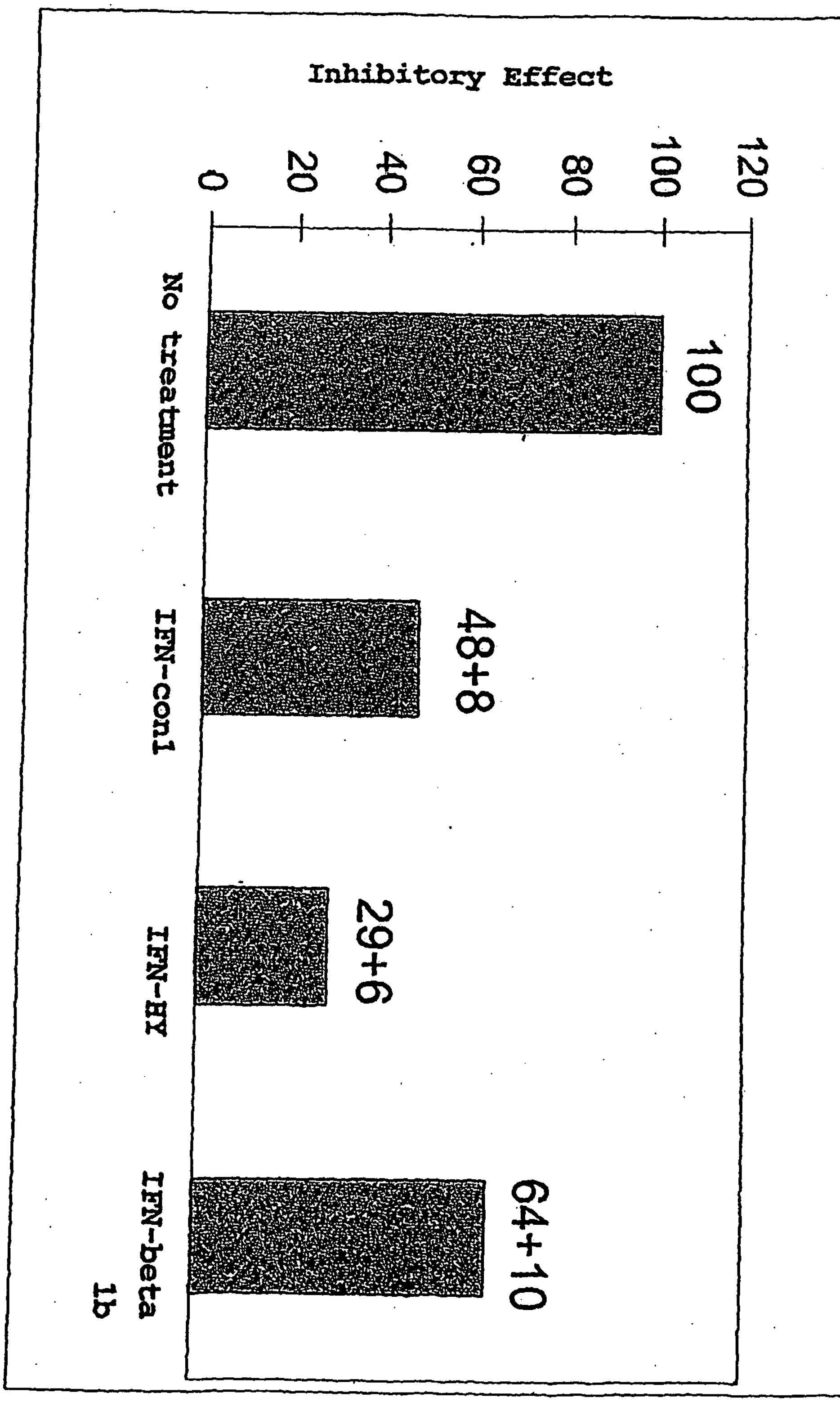
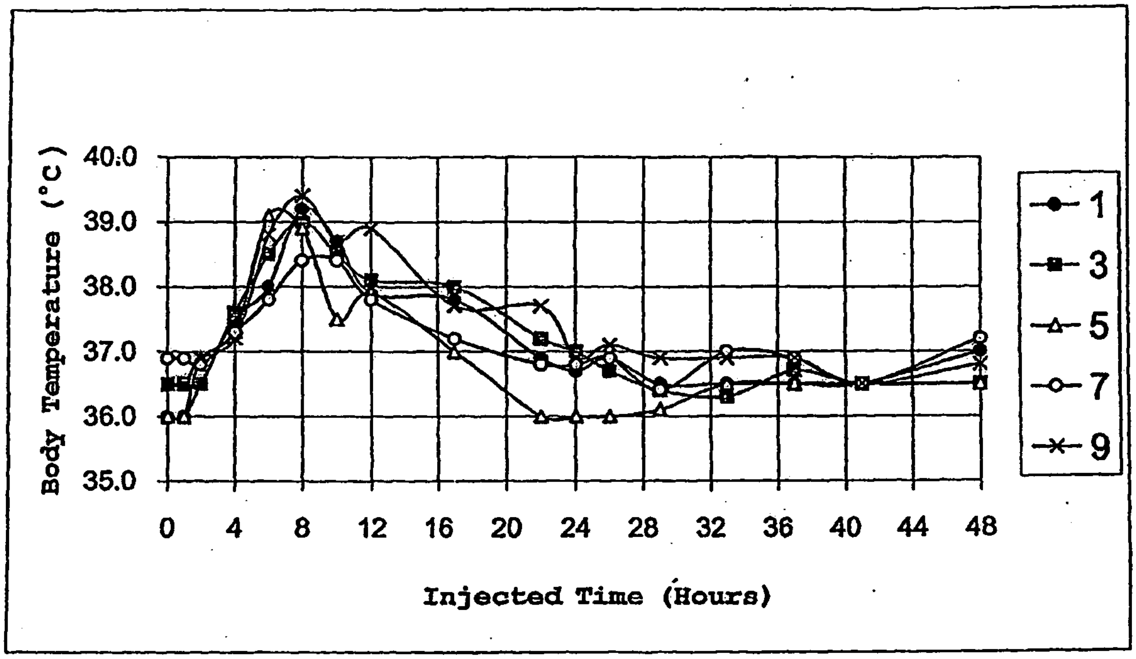

Inhibitory Effect
120
100
80
60
40
20
0
100
48+8
29+6
64+10
No treatment
IFN-con1
IFN-HY
IFN-beta 1b

Figure 3

Figure 4A-1


Body Temperature (°C)
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39.0
38.0
37.0
36.0
35.0
0
4
8
12
16
20
24
28
32
36
40
44
48
Injected Time (Hours)
1
3
5
7
9

Figure 4A-2

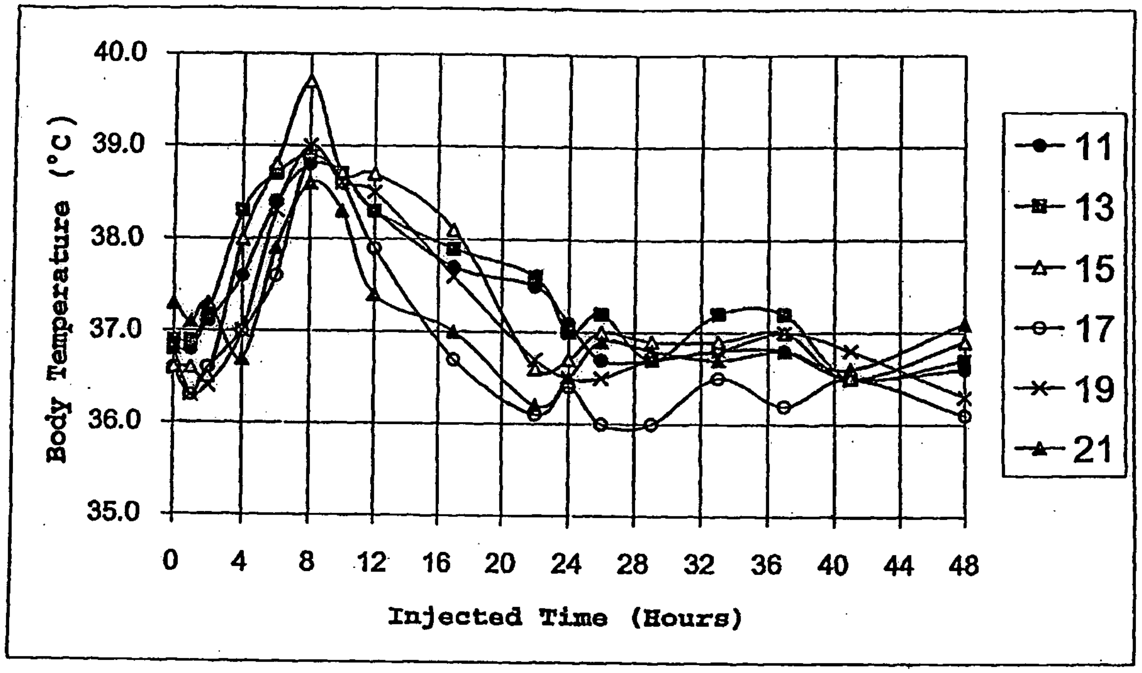

40.0
39.0
38.0
37.0
36.0
35.0
Body Temperature (°C)
0
4
8
12
16
20
24
28
32
36
40
44
48
Injected Time (Hours)
11
13
15
17
19
21

Figure 4B-1

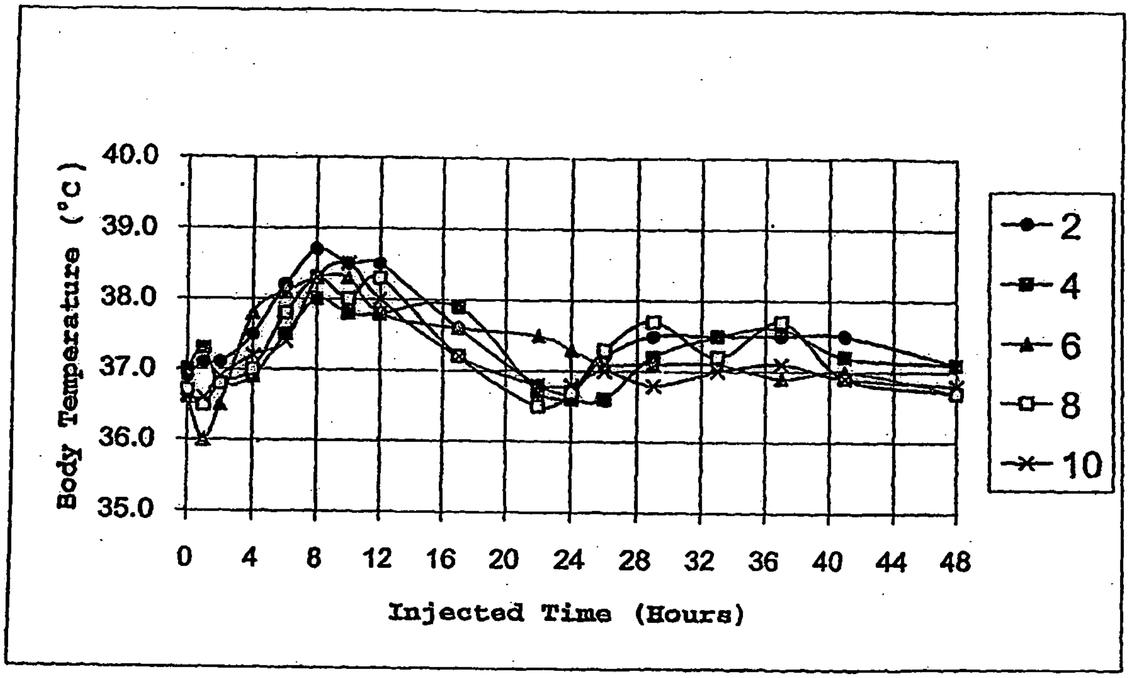
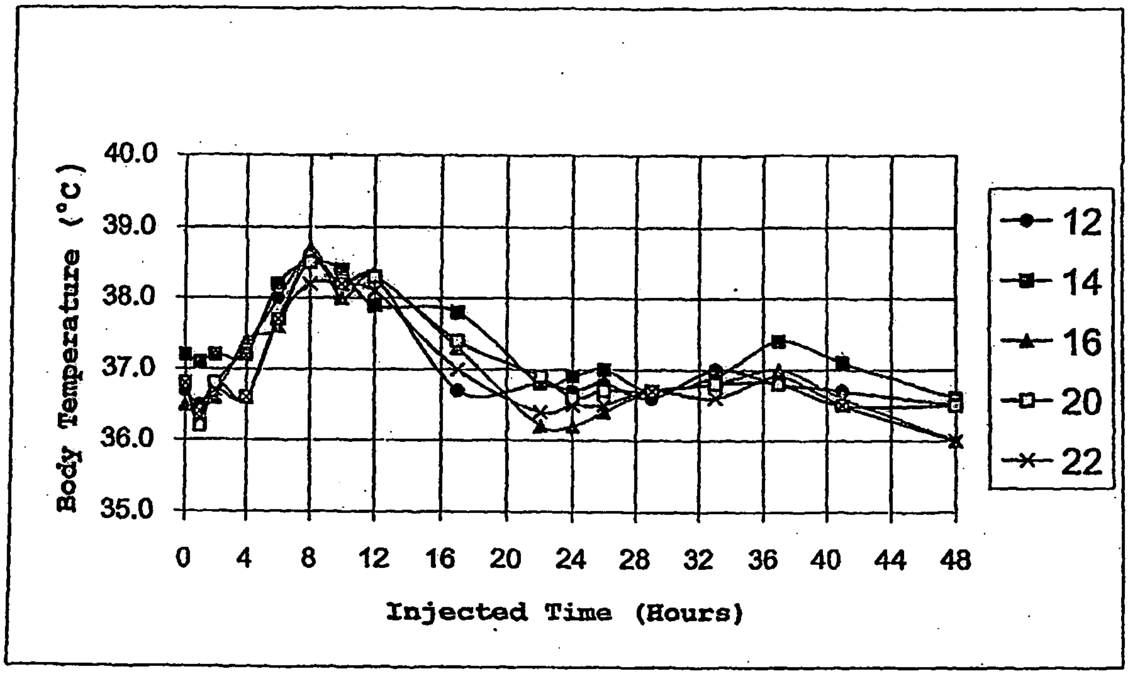

Body Temperature (°C)
40.0
39.0
38.0
37.0
36.0
35.0
0
4
8
12
16
20
24
28
32
36
40
44
48
Injected Time (Hours)
2
4
6
8
10

Figure 4B-2


Body Temperature (°C)
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39.0
38.0
37.0
36.0
35.0
0 4 8 12 16 20 24 28 32 36 40 44 48
Injected Time (Hours)
12
14
16
20
22

Figure 6

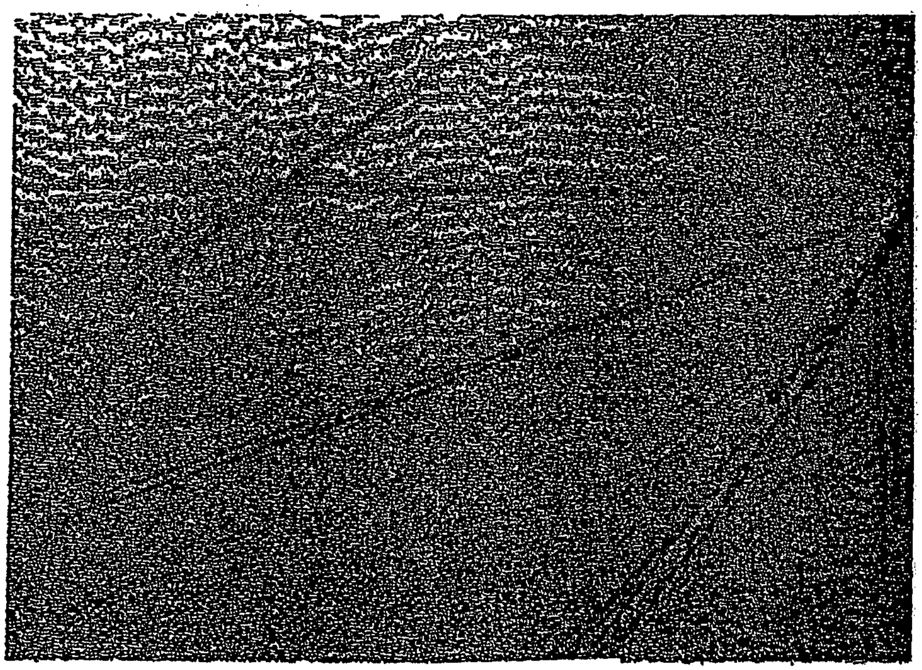


Figure 5

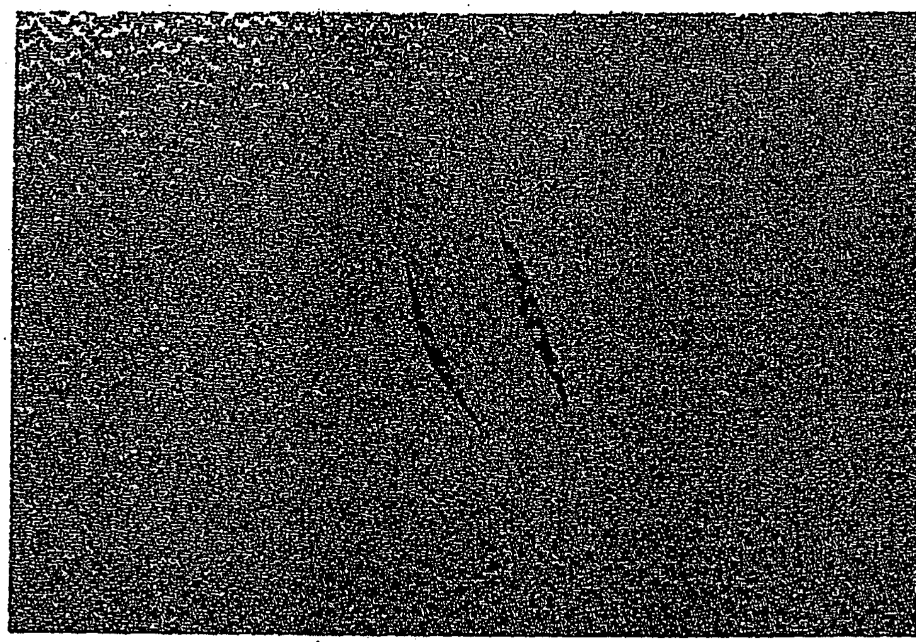
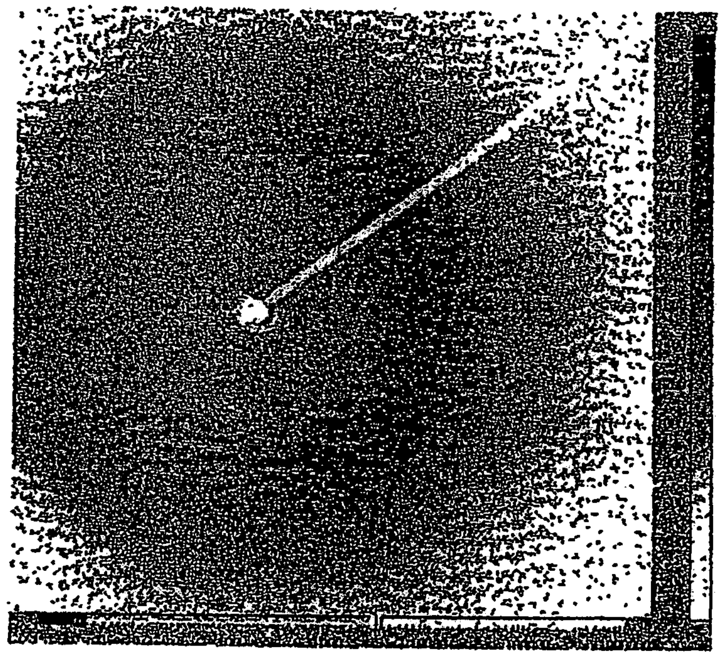


Figure 7



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